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Short-Term Photoinhibition Induces Long-Term Hydrogen Photoproduction in a Phototrophic Culture of *Chlorella sorokiniana* on Complete Medium

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Abstract—This work demonstrates, for the first time, capacity of the *Chlorella sorokiniana* immobilized in alginate to produce hydrogen (H_2) over an extended period of time when cultivated under strictly photoautotropic conditions on complete mineral medium. In order to reduce photosynthetic activity, immobilized cells were subjected to a 30-minute pre-incubation period at high light intensity of 1000 μmol photons $m^{-2}\cdot s^{-1}$. The ability to produce H_2 was evaluated under illumination of 40 μmol/($m^2\cdot s$). The culture not bubbled with argon produced H_2 for 9 days; total gas yield was 0.1 mol H_2/m^2. In the culture under argon atmosphere, the release of H_2 continued for 51 days, resulting in a total yield of 0.55 mol H_2/m^2. The immobilized culture was capable of H_2 production at 16% O_2 in the gas phase, which may be due to the effects of photoinhibition and activation of oxygen uptake pathways in mitochondria and chloroplast. Analysis of the functioning of electron-transport chain in the microalgae cells revealed decrease in the rate of electron transport, increase in the size of the PSII antenna, and development of non-photochemical quenching processes, while activity of PSII remained moderately high (Fv/Fm = 0.4-0.6). Inhibitor analysis using 10^{-5} M DCMU demonstrated that contribution of PSII to hydrogenase reaction increased from 30% on the first day of the experiment to 50% by the fourth day. Addition of 10^{-5} M DBMIB led to the 90% reduction in the rate of H_2 formation on both day 1 and day 4.

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Keywords: green microalgae, primary reactions of photosynthesis, hydrogen photoproduction, photoinhibition, hydrogenase

INTRODUCTION

Molecular hydrogen (H_2) has the highest specific energy capacity among all known types of fuel. Its combustion does not produce carbon, which makes hydrogen an ecologically clean source of energy. Moreover, H_2 plays a key role in a number of industrial processes such as production of methane, methanol, and ammonia [1].

Use of cyanobacteria and green algae for production of H_2 is considered as one of the areas of biotechnology aimed at solving global energy crisis problem. These organisms have unique ability to convert energy of light into H_2 using photosynthesis. Theoretically, efficiency of transformation of solar light into H_2 could reach 10-13% for algae and 6% for nitrogen-fixing cyanobacteria. However, in practice the levels of efficiency of H_2 production by photosynthesizing organisms observed under laboratory settings are significantly lower [2]. This emphasizes the need to further investigate mechanisms of these processes, as well as to develop new technologies to improve efficiency.

Photoproduction of H_2 by green algae occur with participation of the enzyme [FeFe]-hydrogenase, which is synthesized and perform its reaction under anaerobic conditions in chloroplast stroma. This enzyme utilizes electrons generated in the course of water

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photolysis in photosystem II (PSII) and reduces protons to molecular H_2. Activation of hydrogenase play a crucial role for maintenance of continuous photosynthetic electron flow under anaerobic conditions [2-6].

Two independent studies demonstrated the possibility of expression of the hydrogenase gene and its protein synthesis in the green microalgae under anaerobic conditions when photosynthetic activity was reduced [7, 8]. It was also shown that the switch from dark aerobic to light conditions resulted in the decrease of H_2 production mainly due to activation of Calvin cycle, which effectively competes with hydrogenase for electrons [9]. In the process, hydrogenase activity is irreversibly suppressed by O_2 only 4 min after cessation of H_2 release. More over, new strains of *Chlorella vulgaris* have been identified that were capable of H_2 production under aerobic conditions and continuous illumination [10]. The presented results allow considering revision of the existing notions on fast inhibiting effects of O_2 on activity of [FeFe]-hydrogenase *in vivo*. The mechanisms of this phenomenon are poorly understood at present and require further investigation. It could be suggested that tolerance of hydrogen photoproduction to O_2 is due to ability of microalgae to maintain microanaerobic conditions in chloroplast due to partial decrease of the rate of photosynthesis and simultaneous increase of the rate of alternative pathways of electron transfer in chloroplasts, including photorespiration and Meler's reaction [7]. Activity of PSII and the rate of mitochondrial respiration, which change significantly depending on cultivation conditions, also play an important role.

It is well known that sulfur starvation efficiently stimulates long-term H_2 photoproduction by algae under illumination [11, 12]. However, this method, similar to other types of biogenic starvation [13-17], has its limitations: insufficient yield of H_2 for industrial scale and short lifespan of algae. Alternative approaches to stimulate photoproduction of H_2 by microalgae during cultivation in completer nutrient medium have been suggested in the recent decades. Development of such approaches could facilitate increase of the algae lifespan and, as a consequence, prolongation of the period of gas release. In particular, the possibility of prolonged generation of H_2 by the *C. reinhardtii* cells through modification of photosynthetic electron transport has been demonstrated [18, 19]. Furthermore, an innovative method of H_2 photoproduction based on pulsed illumination regime has suggested [8], however, its efficiency is limited by the short period of H_2 release (several days).

Use of immobilized algae cells for production of H_2 under illumination is a promising approach with several advantages. Immobilization of cell culture improves their stability and resistance to external factors, slows down their metabolism (while activity of the enzymes remains high), and allows reaching higher cell density, could facilitate more uniform illumination of cells in photobioreactor, and does not limit gas exchange [20-22]. All these factors taken together results in the increase of yield and duration of H_2 production.

Majority of the studies on production of H_2 were conducted with photoheterotrophic cultures, i.e., cultures with added acetate to the nutrient medium. Acetate serves as an alternative source of carbon, energy (ATP), and as a reducing agent (NADH), providing, in particular, a reducing agent for mitochondrial respiration stimulating transition of the system to anaerobic conditions. However, its use increase significantly costs of H_2 production. From economical point of view the use of photoautotrophic cultures that do not need additional organic substrates seems more promising, which was successfully demonstrated with sulfur-starving culture *C. reinhardtii* as an example [23, 24].

In this study we suggest a protocol ensuring prolonged photoproduction of H_2 by the *C. sorokiniana* cells. This protocol is the first to combine advantages of the approaches discussed above; in particular, it involves use of short-term photoinhibition as a stimulating factor, immobilization of microalgae in alginate films to ensure stability of the process, and photoautotrophic cultivation in complete mineral medium.

MATERIALS AND METHODS

Cultivation of algae. *C. sorokiniana* (isolated from waters of the White Sea, Russia, GenBank ID: KC678067) [25] were cultivated in a HS mineral medium [26] in 300-ml Erlenmeyer flasks at 24°C under continuous illumination. Photon flux density (PFD) 100 μmol photons·m^{-2}·s^{-1} with stirring rate 120 rpm on an ELMI shaker (ELMI, Latvia).

Immobilization of algae. Immobilization of algae in alginate films under sterile conditions was carried out according to the protocol developed by Kosourov and Seibert [20] with some modifications. Algae were precipitated by centrifugation (3000 rpm, 10 min) followed by mixing the obtained precipitate with aqueous solution of sodium alginate (Fluka, USA) at a ratio of 1 ml of 4% alginate solution per 1 g of algae wet weight. The obtained suspension was spread evenly across a mosquito net. Alginate polymerization was induced by spraying films with 50 mM $MgCl_2$ solution. Next films were cut into strips with size 1×3 cm. Each strip was placed into hermetically sealed vials with volume 18 or 68 ml filled with 10 ml of HS medium.

Photoinhibition and cultivation of immobilized cultures. Immobilized algae in hermetically sealed

vials with volume 18 ml or 68 ml were subjected to a single 30-min period of illumination with light of intensity 1000 μmol photons·m^{-2}·s^{-1}. Next 68-ml volume vials were bubbled with an argon flow 30 min (at room light). Next, cultures in both types of vials were incubated from 24 h to 51 days under continuous illumination with PFD 40, 100, or 200 μmol photons·m^{-2}·s^{-1} depending on experimental conditions at room temperature. Culture vials of smaller volume not bubbled with argon flow were used for faster establishing of anaerobic conditions.

Inhibitory analysis. To investigate impact of photosynthesis inhibitors on hydrogen production DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea, diuron) and DBMIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone, dibromothymoquinone) were used at final concentration 10^{-5} M.

Determination of chlorophyll and starch content. Immobilized algae were treated with 0.05 M NaEDTA followed by intensive stirring to complete destruction of alginate matrix. Next cells were precipitated with centrifugation (3000 rpm, 10 min), 6 ml of 90% ethanol was added to the precipitate followed by incubation for 1 day in a fridge. Quantitative evaluation of extracted chlorophyll (Chl) was carried spectrophotometrically [27].

After Chl extraction starch content was determined according to the method described by Gfeller and Gibbs [28] with some modifications. Cell precipitate was washed twice from ethanol by centrifugation (3000 rpm, 10 min) in 1 ml of sodium acetate solution (100 mM, pH 4.5). Next cells were suspended 1 ml of sodium acetate and transferred into sterile centrifuge tubes, which was followed by treatment in an autoclave for 10 min at 1 atmosphere. After cooling the samples to room temperature amyloglucosidase (Sigma, USA) was added to each tube at the amount corresponding to 2 activity units and incubated in a thermostat at 55°C for 18-20 h. Next fluid volume was adjusted to 2 ml with sodium acetate solution and centrifuged. Glucose content in a supernatant was determined spectrophotometrically using glucose oxidase – peroxidase reagent kit (Sigma).

Recording Chl fluorescence. Induction curves of Chl fluorescence (OJIP transients) were recorded with a PEA fluorimeter (Hansatech, United Kingdom). Chl fluorescence was initiated with red light (λ = 650 nm), PFD 1500 μmol photons·m^{-2}·s^{-1}. Efficiency of photochemical transformation of energy in PSII (Fv/Fm) was calculated using the following equation: Fv/Fm = (Fm − Fo)/Fm, where Fo and Fm – minimal and maximal fluorescence values in samples adapted to dark. OJIP-transients were analyzed with the help of JIP-test [29, 30] using equation and parameters presented in Table 1. Immobilized cultures were dark-adapted for 15 min in vials, after which the sample was removed and placed in the fluorescence measuring clump.

Gas chromatography. Content of H_2 was measured with the help of a LKhM-80 gas chromatograph (Russia). Argon flow rate used as a carrier gas was 70 ml/min. Column thermostat temperature was set at 60°C, electric current in katharometer was 70 mA. Quantitative determination of H_2 was carried out with the help of calibration curve constructed based on analysis of the mixtures of argon (99%) and H_2 (1%) in the range of volumes from 50 to 350 μl. Calibration for O_2 was carried out using air samples with volumes from 50 to 350 μl.

Statistical analysis. Three biological replicates were performed for each type of experiment. To evaluate statistical significance of differences One-way ANOVA was used together with Tukey test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Effect of light intensity on activity of PSII and yield of H_2. In the beginning of the study the effects of light intensity on activity of PSII were evaluated in order to determine the level of illumination that causes significant decrease of the PSII activity but without its complete inactivation. The obtained data (Table 2) indicate that the highest decrease of the

Table 1. List of equations used in JIP-test with explanations

Parameters of JIP-test	Equation for calculation	Interpretation of the parameter
M_o	$4(F_{300\mu s} - F_o)/(Fm - Fo)$	initial slope of induction curve
V_j	$(F_{2ms} - Fo)/(F_m - Fo)$	relative variable fluorescence at 2 ms
ET_o/RC	$M_o \cdot (1/V_j) \cdot (1 - V_j)$	electron flow through RC
DI_o/RC	$ABS/RC - TR_o/RC$	energy scattered energy by RC
ABS/RC	$M_o \cdot (1/V_J) \cdot (1/(Fv/Fm))$	energy absorbed by RC

Table 2. Effect of light intensity on Fv/Fm and H2 yield in immobilized cells of *C. sorokiniana*

Intensity of moderate (40) and photoinhibiting light, μmol photons·m^{-2}·s^{-1}	Fv/Fm	Intensity of illumination after the action of photoinhibiting light (PFD 1000), μmol photons·m^{-2}·s^{-1}	Yield of H_2, mmol/m^2
40	0.704 ± 0.02	40	3.80 ± 0,5
500	0.448* ± 0.03	100	1.14* ± 0.2
1000	0.253* ± 0.02	200	0.76* ± 0.2

Note. Fv/Fm measurement were conducted 30 min after incubation of cultures under illumination with light of intensity 40, 500, and 1000 μmol photons·m^{-2}·s^{-1}. Prior to measuring Chl fluorescence samples were adapted in the dark for 15 min. Yield of H_2 was determined after 24-h incubation of the cultures placed in the 18-ml volume vials not bubbled with argon under light intensity 40, 100, and 200 μmol photons·m^{-2}·s^{-1}. Initial concentration of Chl ~ 32 μg per strip. * Significant difference from the values obtained with illumination of 40 μmol photons·m^{-2}·s^{-1}, $p < 0.05$.

efficiency of photochemical energy conversion in PSII (parameter Fv/Fm) was recorded at the level of illumination of 1000 μmol photons·m^{-2}·s^{-1}. In this case the Fv/Fm value decreased by 64% in comparison with the initial level (from 0.70 to 0.25).

In the second part of the study effects of the light intensity on efficiency of H_2 production by the immobilized cultures were examined. First the samples were subjected to the action of high-intensity light (1000 μmol photons·m^{-2}·s^{-1}) for 30 min, and next the samples were incubated for one day under illumination 40, 100, and 200 μmol photons·m^{-2}·s^{-1}. Maximal amount of released H_2 (3.8 mmol/m^2) was recorded at PFD of 40 μmol photons·m^{-2}·s^{-1}. Increase of illumination to 100 or 200 μmol photons·m^{-2}·s^{-1} resulted in the decrease of H_2 by 70% and 80%, respectively.

The changes of Fv/Fm and content of O_2, H_2, glucose. In the second stage of the study, duration of H_2 release and its amount during incubation of the cultures without replacing air with inert gas and in argon atmosphere were evaluated. As can be seen in Fig. 1, in both cases the cultures released ~0.1 mol H_2/m^2 during 9 days. However, production of H_2 by the cultures not bubbled with argon ceased before this time point. On the contrary, the immobilized algae cultivated in argon atmosphere continued to release H_2 for 51 days. Yield of H_2 was 0.55 mol H_2/m^2.

Further study concentrated on the culture not bubbled with argon, because this approach does not involve use of expensive argon. Content of O_2 in the gas phase of vials decreased gradually from 20% to 9% during the first 8 days of the experiment (Fig. 1a). At the 9th day concentration of O_2 increased to 14% and remained at this level until the end of experiment.

To evaluate photosynthetic activity of the algae the Fv/Fm parameter was used. Value of the Fv/Fm parameter decreased from 0.7 to 0.25 after initial photoinhibition (Fig. 1a). However, already after one day the Fv/Fm parameters was recovered to the level of 0.4, and at the 8th day it reached the value of 0.62. By the 20th day of the experiment the Fv/Fm value reached control levels (0.7), which indicated complete recovery of the PSII activity.

Content of glucose in the cells increased by 30% already after 30 min of photoinhibition and continued to grow increasing more than 5-fold in comparison with the control level at the 4th day of the experiment. However, the glucose content decreased from 1.13 ng to 0.36 mg on the 5th day of the experiment followed by the decrease in the next days reaching minimum value of 0.1 mg at the 13th day of the experiment. This was followed by the gradual increase of the glucose content up to control levels (0.2 mg).

Analysis of OJIP-transients. Fluorescence induction curves (OJIP-transients) were recorded and analyzed to investigate functioning of photosynthetic electron transport chain (ETC). Typical curves obtained with the immobilized cultures not bubbled with argon at the days 1, 4, 8, and 20 of the experiment are presented in Fig. 2. Typical curve recorded in the control culture with all phases of fluorescence growth (OJ, JI, and IP) clearly visible, is shown in the inset. The OJIP-curves recorded at different days of the experiment differed significantly in the variable fluorescence (OP). Minimal OP value was observed at the first day of the experiment, with amplitude of the OJ phase contributing significantly to the variable fluorescence, while the JIP phase was only weakly pronounced. Gradual increase of OP was observed in the following days, which indicates growth of the number of active centers in PSII. The JIP phase became clearly visible on the 4th day of the experiment.

OJIP-transients were analyzed using JIP-test, which allows evaluation of the processes of transformation of light energy in photosynthetic reactions (Table 3). The Vj parameter, reflecting the portion of closed RC of PSII uncapable of transfer electron from Q_A (primary quinone acceptor in PSII) to further ETC, was 50% higher than in the control group during first four day of the experiment. On the day 8 this parameter

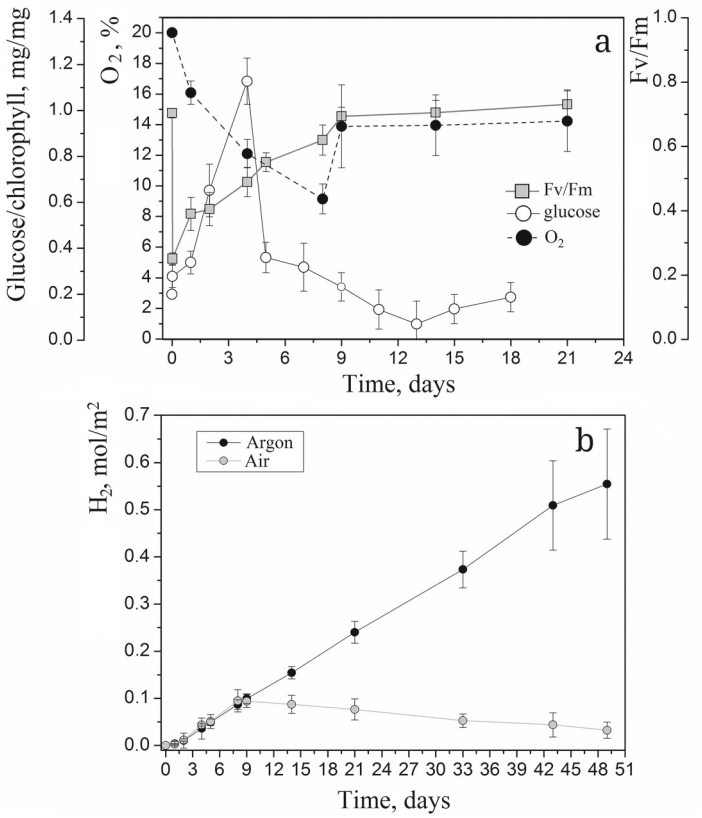

Fig. 1. Effect of gas phase composition on metabolism and release of hydrogen. Change of content of O_2 in gas phase, of glucose in the cells, and of activity of PSII (Fv/Fm) in immobilized algae not bubbled with argon (a). Effect of gas phase composition on release of H_2 by *C. sorokiniana* (b). Algae were cultivated in argon atmosphere (black circles) and without replacing air with inert gas (gray circles). Cultures were incubated in complete HS-medium under illumination with 40 μmol photons·m^{-2}·s^{-1} light. Initial concentration of Chl ~ 32 μg per strip. Results of a typical experiment are presented demonstrating average value of H_2 yield calculated based on the data obtained for 3 vials. Experiments with duration 49 days was repeated twice, and with 21 days – three times.

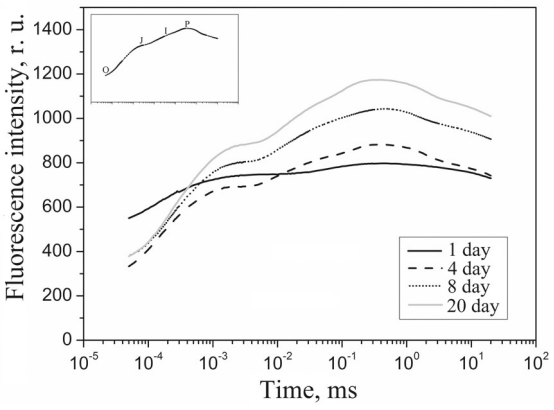

Fig. 2. Typical OJIP-transients recorded with immobilized cultures of *C. sorokiniana* on the 1st, 4th, 8th, and 20th day after photoinhibition. Algae were cultivated under conditions without air replacement with inert gas in complete HS medium and illumination with 40 μmol photons·m^{-2}·s^{-1} light. Inset shows a typical curve recorded for immobilized culture not subjected to photoinhibition, which served as a control. OJIP transients were recorded one hour after the end of immobilization and incubation under conditions described above.

remained higher by 25% than in the control, and by the day 20 it returned to the control values. The rate of electron transport to RC (ETo/RC) remained by 60-70% lower than in the control over the entire duration of the experiment. The parameter ABS/RC, which reflects size of antenna, increased more than 6-fold in the first day. In the following days there was an insignificant decrease of this parameter, but on the day 8 it was still 4-fold higher, and at the day 20 – almost 3-fold higher than the control level. The DIo/RC parameter characterizing development of non-photochemical quenching increased more than 9-fold in the first day of the experiment. This was followed by its gradual decrease, however, by the day 20 this parameter was still 2-fold higher than in the control.

Effect of DCMU on H_2 evolution. Effect of addition of 10^{-5} M DCMU on the rate of H_2 production (a) and total yield of H_2 (b) are shown in Fig. 3. In the control the rate of H_2 generation on the days 1 and 2 of the experiment was 12.5 μmol H_2 mg^{-1} Chl h^{-1}. Addition of DCMU resulted in the decrease of the rate of H_2 production by 30% during the first day.

Table 3. Parameters of JIP-test calculated based of the fluorescence data obtained from the OJIP-curves

Parameters of JIP-test	Control	Day 1	Day 4	Day 8	Day 20
Vj	0.52 ± 0.03	0.79* ± 0.02	0.70* ± 0.04	0.65* ± 0.03	0.54 ± 0.05
ABS/RC	0.92 ± 0.21	5.63* ± 0.16	4.69* ± 0.25	3.70* ± 0.34	2.63* ± 0.25
DIo/RC	0.35 ± 0.10	3.35* ± 0.11	2.40* ± 0.18	1.66* ± 0.18	0.71* ± 0.16
ETo/RC	1.61 ± 0.42	0.47* ± 0.51	0.58* ± 0.48	0.63* ± 0.43	0.68* ± 0.45

Note. Mean values based on 3-5 replicated are presented. * Significant difference from the control, $p < 0.05$.

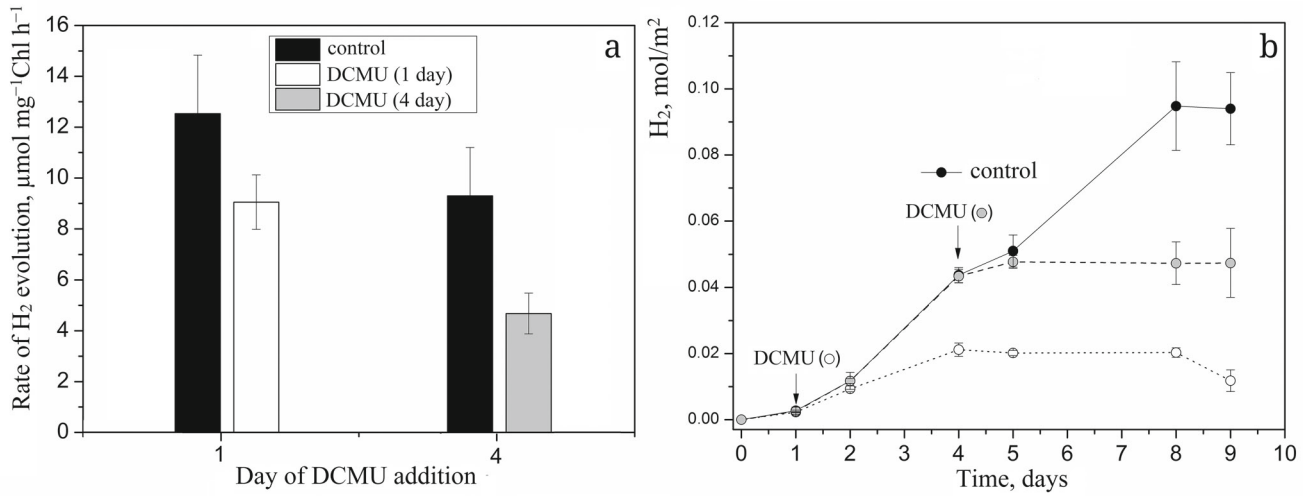

Fig. 3. Effect of 10^{-5} M DCMU on the rate of H_2 formation (a) and total yield of H_2 evolution (b) in the immobilized *C. sorokiniana* cells cultivated under conditions without replacement of air with inert gas.

After addition of the inhibitor release of H_2 continued up to the day 4 of the experiment, however, total yield of the gas was by 50% lower in comparison with the control. The rate of H_2 production in the control from the day 4 to the day 5 of the experiment was 9.05 μmol H_2 mg^{-1} Chl h^{-1}, and addition of DCMU on the day 4 resulted in the decrease of the rate by 50%. Accumulation of H_2 in the gas phase of vials was observed only during the first day after inhibitor addition, and after that the process completely stopped. Addition of 10^{-5} M DBMIB both in the first and fourth day of the experiment inhibited formation of H_2 by 90% (data not shown).

DISCUSSION

The suggested approach – induction of long-term photoproduction of H_2 – facilitated release of 0.10 mol H_2/m^2 by the immobilized photoautotrophic *C. sorokiniana* culture cultivated in the complete medium for 9 days in a natural atmosphere, and 0.55 mol H_2/m^2 during 51-day cultivation in argon atmosphere. Reaching these levels of H_2 production was possible due to the use of protocol involving initial preillumination of immobilized algae with photoinhibiting light with intensity 1000 μmol photons·m^{-2}·s^{-1} for 30 min followed by cultivation under light intensity of 40 μmol photons·m^{-2}·s^{-1}.

The method of photoinhibition of algae has been used previously for production of H_2. In particular, it was shown that the phototrophic culture *Chlamydomonas reinhardtii*, which was pre-treated with nitrogen bubbling, is capable of long-term (96 h) production of H_2 under illumination of 320 μmol photons·m^{-2}·s^{-1}. Total yield was 227 μl of H_2 per ml of culture. To remove the released O_2 a sorbent based on iron salts was used [31]. An approach has been suggested based on short-term photoinhibition when the *C. reinhardtii* culture grown on the medium with acetate was subjected to the short-term (15-30 min) illumination with high light (2000 μmol photons·m^{-2}·s^{-1}). To facilitate anaerobic conditions the culture was additionally bubbled with nitrogen. The following illumination with 15 μmol photons·m^{-2}·s^{-1} light resulted in production of H_2, which, however, continued no more than 5 h [32].

In this study we concentrated on investigation of photoproduction of H_2 by the algae cultivated

in the air. This approach allows avoiding the use of costly inert gases, which is significant from economical point of view. Concentration of O_2 in the composition of gas phase in vials decreased from the initial 20.9% to 9% by the day 9 of the experiment (Fig. 1a). Remarkably, release of H_2 was observed at O_2 content in the gas phase of 16%, which could be explained by activation of alternative electron transport chains. Such processes as Meler reaction, photorespiration, chlororespiration, and reactions with participation of flavoproteins could results in formation of microanaerobic areas in chloroplast, thus protecting hydrogenase against inactivation by O_2 [2, 4, 7, 33]. It must be mentioned that immobilized cells are arranged in layers, which could cause differences in physiological states of algae depending on their location. The surface cells are in contact with external O_2, while the cells in inner layers could be protected form its effects.

In the time period from day 1 to day 8 of the experiment the PSII activity (Fv/Fm) was moderately high (0.4-0.6). However, significant fraction of the reaction centers in PSII (Vj) were in the closed state during first 4 days (Table 3). Moreover, electron flow to RC (ETo/RC) was lower by 60% in comparison with the control. At the same time, significant increase of antenna size (ABS/RC) was observed, as well as development of the processes of non-photochemical quenching (DIo/RC). Increase of the antenna size could be due to the self-shading effect emerging because of high density of immobilized cells in the alginate layers.

It is known that hydrogenase receives electrons both from PSII and from reducing equivalents formed during starch degradation, which are transferred to ETC via pool of quinones involved in process of chlororespiration [2-4]. To determine contribution of PSII-dependent and PSII-independent pathways to production of H_2, an inhibitory analysis was conducted on the 1st and 4th days of the experiment. Addition of 10^{-5} M DCMU that blocks electron flux at the level of Q_a-centers of PSII, decreased the rate of H_2 release by 30% in the first day of incubation (Fig. 3a). Hence, PSII was a minor donor of electrons in comparison with the electron flux from chloroplast stroma to the pool of plastoquinones. Addition of DCMU on the 4th day of experiment, when sharp decrease of glucose content was observed, resulted in 50% decrease of the rate of H_2 production indicate growth of PSII contribution to hydrogen photoproduction. Addition of DBMIB that inhibits electron transport at the level of cytochrome b_6f-complex (i.e., blocking electron flux both from PSII-dependent and PSII-independent pathways) both on the day 1 and day 4 of the experiment resulted in the significant (90%) decrease of the rate of H_2 production. The obtained results indicate that the electrons coming from PSII and from the processes of starch degradation play a key role in generation of H_2 in *C. sorokiniana* under the used experimental conditions.

Differences in contribution of PSII to production of H_2 on the first and fourth day of the experiment could be due to the changes in activity of Calvin cycle. Gradual increase of the glucose level inside the cells was revealed in the course of our study (Fig. 1a). Nevertheless, significant (68%) decrease of the glucose level was observed from the day 4 to the day 5 of the experiment. This fact could be explained by different activity of the reactions of Calvin cycle, which is the main process competing with hydrogenase reaction, activity of which could cause termination of H_2 production prior to inactivation of hydrogenase by oxygen [9].

CONCLUSIONS

Photoproduction of H_2 by green microalgae is a promising direction for biotechnology research. Despite the achieved progress in this area, many issues remain unresolved. One of the key problems is development of economically profitable process capable of ensuring long-term production of H_2 by algae cells.

In this study we suggest the method for stimulation of hydrogen photoproduction in the green microalgae cells based on combination of photoinhibition induced by short-term illumination of algae cell with high-intensity light, immobilization of microalgae in alginate, and photoautotrophic cultivation of cells in complete mineral medium. This approach allows achieving long-term production of H_2 up to 9 day in the natural air environment and up to 51 days in argon atmosphere with simultaneous decrease of the costs of the process. In our experiments total yield of hydrogen was 0.55 mol H_2/m^2 in the case of incubation in argon atmosphere, which is equivalent with regard to Chl to 16.7 mmol H_2 mg Chl^{-1} m^{-2}. This result exceeds the previous reported for the free (non-immobilized) *C. reinhardtii* cells, which released H_2 for more than 40 days during incubation in the medium with acetate, but total yield did not exceed 2 mmol H_2 Chl^{-1} (0.33 mmol H_2 mg Chl^{-1} l^{-1}) [18]. It is important to note that it is the longest duration of H_2 generation reported in the literature.

We also demonstrated in this study that the culture not-bubbled with argon released 0.1 mol H_2 m^{-2} for 9 days. For comparison, the sulfur-starving photoheterotrophic culture of *C. reinhardtii* in air atmosphere and with similar concentration of Chl releases 0.04 mol H_2/m^2 [20]. Under strict photoautotrophic conditions the non-immobilized sulfur-starving *C. reinhardtii* cells were able to release around 3 mmol H_2 Chl^{-1} [24].

The obtained results show that the method has competitive potential, which could be realized with optimization of cultivation conditions. However, further optimization of the method requires understanding of the mechanisms of the observed glucose content changes in the microalgae cells during incubation in air atmosphere and associated changes in activity of Calvin cycle and respiration, which would require additional investigations.

Abbreviations. PSII, photosystem II; Chl, chlorophyll; DCMU, (3-(3,4-dichlorophenyl)-1,1-dimethylurea) (diuron); Fv/Fm, efficiency of photochemical energy transformation in PSII.

Contributions. A. A. Volgusheva – concept of the study, conducting experiments, and writing text of the paper; A. A. Volgusheva and T. K. Antal – discussion of the study results; T. K. Antal – editing text of the paper.

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Ethics approval and consent to participate. This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest. The authors of this work declare that they have no conflicts of interest.

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